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TITLE: Integration of Genomic, Biologic, and Chemical Approaches to Target p53 Loss and Gain-of-Function in Triple Negative Breast Cancer

PRINCIPAL INVESTIGATOR: Jennifer A. Pietenpol, Ph.D.

CONTRACTING ORGANIZATION: Vanderbilt University School of Medicine
Nashville, TN 37203-6869

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Jennifer A. Pietenpol, Ph.D

E-Mail: j.pietenpol@vanderbilt.edu

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Vanderbilt University Medical Center
Office of Sponsored Programs
1400 18th Avenue South
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14. ABSTRACT

This is the third annual progress report for DoD Award W81XWH-13-1-0287 / BC123219, investigating biochemical states resulting from alterations in the p53 signaling pathway in triple negative breast cancer (TNBC). Development of therapies for TNBC is a clinical and scientific challenge due to the heterogeneity of the disease and the lack of recurrent, drug-targetable molecular alterations. Our research focuses on the p53 tumor suppressor pathway, which is altered in the majority of TNBC cases and produces two adaptive states: loss of function (LOF) of wild-type p53 through mutation, gene silencing, or amplification of negative p53 regulators, and gain of function (GOF) displayed by some "hotspot" p53 mutant proteins that accumulate to high levels within the cell and drive oncogenic phenotypes including growth, migration, and drug resistance. We hypothesize that targeting these adaptive biochemical states will provide candidate therapeutic targets for a large fraction of TNBC, a cancer for which there are no molecular targets to date. We are pursuing two specific aims: 1) to identify which signaling pathways, in either adaptive state, are required for TNBC cell viability, and 2) to test validated targets for "druggability" by fragment-based screening and develop small molecular inhibitors against targets that are both valid and druggable.

15. SUBJECT TERMS

Nothing listed

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Table of Contents

Page

1. INTRODUCTION

This is our third annual progress report for DoD Award W81XWH-13-1-0287, investigating biochemical states resulting from alterations in the p53 signaling pathway in triple-negative breast cancer (TNBC). Development of therapies for TNBC is a clinical and scientific challenge due to the heterogeneity of the disease and the lack of recurrent, drug-targetable molecular alterations[1-3]. Our research focuses on the p53 tumor suppressor pathway, which is altered in a majority of TNBC cases and produces two adaptive states: loss of function (LOF) of wild-type p53 through mutation, gene silencing, or amplification of negative p53 regulators, and gain of function (GOF) displayed by some "hotspot" p53 mutant proteins that accumulate to high levels within the cell and drive oncogenic phenotypes including growth, migration, and drug resistance[4-7]. We hypothesize that targeting these adaptive biochemical states will provide candidate therapeutic targets for a large fraction of TNBC, a cancer for which there are no molecular targets to date. We are pursuing two specific aims: 1) to identify which signaling pathways, in either adaptive state, are required for TNBC cell viability, and 2) to test validated targets for "druggability" by fragment-based screening and develop small molecular inhibitors against targets that are both valid and druggable.

2. KEYWORDS

Listed in original application with emphasis on the following in this progress report:

p53
triple-negative breast cancer
subtypes
gene expression
somatic cell genetics
CRISPR/Cas

3. ACCOMPLISHMENTS

Major Goals of Project and Accomplishments

Specific Aim 1: We will verify which clinically characterized p53 mutants are oncogenic in TNBC lines and model systems. TNBC cell lines representative of the different adaptive states will be subjected to high-throughput siRNA-based synthetic lethality screens as a primary search for signaling pathways that, when targeted, can impact viability under a given adaptive state. One sub-aim is to target the p53 LOF adaptive state, and the other is to identify pathways that provide insight to how select high frequency gain of function p53 mutants can confer an oncogenic state. The intent is to identify key pathway components that can be advanced as candidate targets for "druggability."

In the last annual report, we described the creation of an isogenic p53 mutant TNBC cell line panel using CRISPR/Cas-mediated genome editing[8] and the resultant identified chemoresistance in select GOF mutants by high-throughput drug screening. In the present reporting period, we have continued our characterization of this panel, specifically focusing on four genotypes: wild-type, the R175H GOF missense mutant, the R273H GOF missense mutant, and frameshift null mutations leading to a lack of p53 expression (LOF). These isogenic p53 mutant-expressing cell lines have continued to serve as valuable cell context-identical reagents for transcriptional and molecular analysis of the GOF and LOF biochemical states.

Given the differential chemoresistance and other metabolic phenotypes we observed, we conducted transcriptional profiling of the isogenic cell line panel using RNA-seq, followed by differential gene

expression analysis using the R package DESeq2[9]. Count data from aligned RNA-seq reads were transformed and analyzed using a linear model that evaluated the effect of differing p53 genotypes between the isogenic mutants. Genes that were up- or downregulated as a function of genotype were identified as depicted in **Figure 1**.

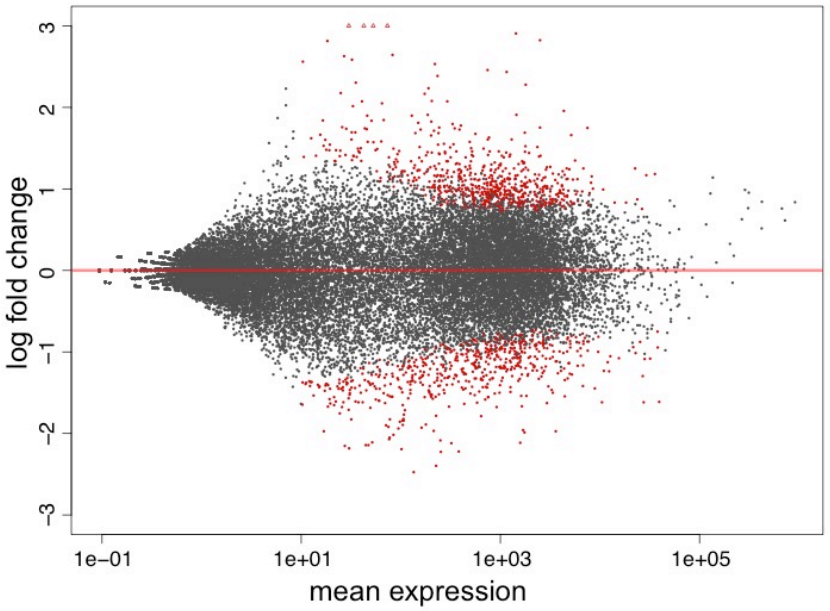

Figure 1. Identification of differentially expressed genes from p53 isogenic RNA-seq data. RNA from two isogenic cell lines of differing p53 genotype was poly-A selected, converted to a stranded RNA-seq library, and sequenced on the Illumina HiSeq 2500. The resultant reads were aligned and counts were obtained for each gene locus based on Gencode annotations. The resulting count data were transformed and plotted for log fold change using the DESeq2 package in R[9]. Genes with an FDR-adjusted p value of 0.1 or less are depicted in red.

RNA-seq and DESeq2 analysis were conducted on 2 biological replicates of each missense mutant genotype as well as wild-type and null controls. Our preliminary analysis identified hundreds of genes that are differentially expressed between phenotypes; these include mutant-specific changes as well as genes differentially expressed between the p53-deficient (mutant and null) and p53-competent (wild-type) backgrounds.

An example of a differentially expressed gene cluster identified by this method is depicted in **Figure 2**. In this instance, a subset of genes in the R273H GOF mutant was identified as being significantly differently expressed compared to the wild-type, R175H GOF, and null LOF genotypes. This GOF mutant-unique transcriptional signature provides numerous opportunities to investigate mechanisms for the chemoresistance phenotype described in the previous progress report and the numerous other GOF phenotypes reported in the literature.

Specific Aim 2: To avoid expending valuable time and resources on targets for which there is a low probability of success for therapeutic intervention, we will clone, express, and purify potential target proteins (identified and validated through synthetic lethal screening in Aim 1) and screen them against a molecular fragment library (~15,000 compounds, <300 M.W.) using two-dimensional heteronuclear single quantum correlation (HSQC) NMR (of uniformly ^{15}N-labeled proteins) or saturation transfer difference (STD) NMR (of unlabeled proteins). Proteins that exhibit binding to >0.1% of the molecules in this screen will be considered druggable and will be candidates for further discovery efforts including modifying the hits obtained in fragment-based screens to produce molecules that bind more tightly to the target protein. These efforts will be guided by NMR and/or X-ray crystal structures of protein-ligand complexes using iterative structure-based design. Compounds will also be optimized for their ability to block the biochemical and cellular functions of the target, leading to an inhibition of growth of cancer cell lines.

Based on preliminary data from the current reporting period, potential target proteins for these experiments may include transcriptional cofactors and downstream gene targets involved in the GOF missense mutant gene expression signatures as depicted in **Figure 2**. Further mechanistic investigation – including knockdown or knockout of these potential targets – will aid in narrowing the target pool for the experiments in this Aim.

Training and Professional Development Provided by the Project to Date & Dissemination of Results to Communities of Interest

This IDEA award has promoted the training and career development of Timothy Shaver, a very talented pre-doctoral fellow in the laboratory. Tim's current dissertation work lies at the intersection of two long-standing areas of investigation in the laboratory: the molecular biology of the p53 tumor suppressive response and the identification of therapeutic targets in cancer. A major focus of his dissertation research is determining the mechanism and "targetability" of a mutant p53-adapted state in triple-negative breast cancer. Tim's preliminary results led to the successful submission for this Department of Defense Breast Cancer Research Program IDEA Award. In addition, on the strength of this research and his record to date, he received an F31 predoctoral NRSA fellowship two years ago. Thus, this IDEA award supports the methods and supplies for the experiments outlined as well as covers the effort and provides the active training of a senior research associate, Hailing Jin.

Based on Tim's progress with the research aims of this award, he attended the International p53 Workshop in Stockholm last summer, and presented abstracts at the 8th International MDM2 Workshop in New Orleans, Louisiana and the 7th International p63/p73 Workshop at Massachusetts General Hospital in April of this year (**Dissemination of Results to Communities of Interest**). Due to the small and focused nature of the meetings, Tim has had the opportunity to discuss his research one-on-one and network with principal investigators and trainees in this exciting field of study.

Plans for the next reporting period to accomplish the goals

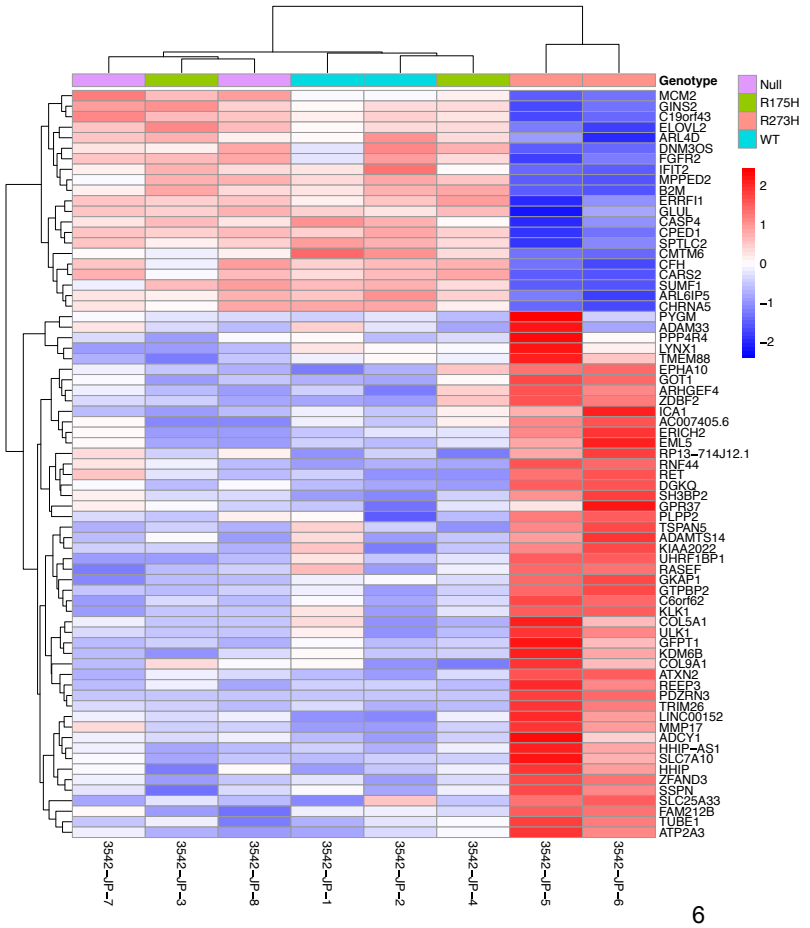

Figure 2. The R273H p53 GOF missense mutant exhibits a "core" set of uniquely expressed genes. Gene locus counts from RNA-seq data were aligned and analyzed as depicted in Figure 1. A linear model evaluating differential gene expression as a function of p53 genotype was used to identify significantly differently expressed genes in pairwise combinations between genotypes. A set of 71 genes was differentially expressed between the R273H GOF mutant and each of the other three genotypes depicted in the legend at top right; the expression values for these genes are plotted above as a clustered, row-normalized heatmap.

Specific Aim 1: Similar to the plans outlined in our recent request for a no-cost extension, our current efforts to identify the mechanism underlying the p53 LOF and GOF-adapted biochemical states includes the following:

RNA-seq: In on our preliminary data, we have observed mutant-specific changes representative of the GOF-adapted state and genes differentially expressed in the null vs. wild-type settings, representative of the LOF-adapted state. We will continue our analysis with three main goals: 1) To corroborate our differential gene expression findings with additional isogenic clones harboring the genotypes of interest. This is critical to rule out the confounding effect of clonal variation on the phenotypes being measured. 2) To identify similarly differentially expressed genes in RNA-seq data from clinical tumor specimens. Our laboratory has extensive experience leveraging publically available sequencing resources such as The Cancer Genome Atlas, which contain transcriptional data from thousands of tumor specimens representing each of the p53 genotypes of interest. Any unique gene expression signatures that are conserved between our *in vitro* model and these clinical specimens will be prime candidates for mechanistic investigation. 3) To identify gene expression changes upon loss of GOF mutant expression. Our candidate gene expression signatures, as depicted in **Figure 2**, are found in the GOF (missense mutant-expressing) state but not in the LOF null state. To validate that mutant p53 is directly responsible for this altered transcription, we will use the same CRISPR-mediated genome editing techniques successfully used to originally create the isogenic cell line panel to knock out the GOF mutants. RNA-seq or qPCR pre- and post-knockdown should identify transcripts whose expression is dependent on the expression of mutant p53.

ChIP-seq: We have performed chromatin immunoprecipitation followed by next-generation DNA sequencing (ChIP-seq) on multiple isogenic cell lines to identify genomic binding sites of the p53 proteins in each genotype. We will extend this analysis to the remainder of the isogenic cell line panel, and we will additionally evaluate well-characterized epigenetic marks such as histone methylation and acetylation by ChIP-seq. Correlation of these data sets with the RNA sequencing described above will allow identification of direct and indirect p53 transcriptional targets in the different p53 GOF and LOF contexts represented by our isogenic cell lines.

IP-MS: We have conducted immunoprecipitation (IP) of wild-type p53 followed by mass spectrometry (MS) to optimize the identification p53 protein-protein interacting partners. In the requested no-cost extension period, we will make use of a quantitative proteomic method known as SILAC (stable isotope labeling with amino acids in cell culture) to quantify changes in protein interaction between the wild-type and mutant p53 isogenic cell lines; the accompanying frameshift null cell lines will serve as an effective negative control for the effects of nonspecific immunoprecipitation, thereby enriching the resulting target list. SILAC and other techniques have been successfully used by other groups to identify mutant p53 interacting partners in the context of transient overexpression; using long-term cultured isogenic cell lines expressing p53 mutants from the endogenous allele, we expect more physiologically relevant findings from this experiment.

Specific Aim 2: As noted in the previous annual report, the most promising candidates from each sub-aim of Specific Aim 1 (LOF vs. GOF adaptive states) will be advanced to small molecule screening to evaluate their potential for druggability and to identify promising lead molecules for pharmaceutical development. Using a “fragment library” of 10,000 small molecules with a molecular weight of 280 or less, we will conduct NMR screens to assess protein-ligand interactions. Proteins with hit rates >0.1% will be undergo a fragment-based drug design protocol, as outlined in our original proposal, in addition to screening with more traditional chemical libraries. Lead optimization and cellular evaluation will be conducted on an ongoing basis once candidate protein-ligand partners are

identified. In particular, we expect the candidate target proteins to be involved in mutant-specific transcriptional profiles (as depicted in **Figure 2**), as co-activators or as gene targets.

In sum, the above experiments will provide a comprehensive, multidimensional dataset detailing the biochemical state accompanying the p53 wild-type, missense mutant (GOF), and null (LOF) settings. In the next reporting period, we will move key findings from analysis of this dataset to publication and begin dissemination of our novel reagents to the academic community.

4. IMPACT – KEY RESEARCH ACCOMPLISHMENTS

- Generation of over 16 isogenic clones expressing three of the highest-frequency p53 GOF mutants found in TNBC, along with wild-type and LOF controls. Partial RNA-seq and ChIP-seq genomic characterization of all the cell line models. Once we have fully characterized these models and results are peer-reviewed, these reagents and genomic data sets will be available to the scientific community.

- Determination of half-maximal inhibitory concentration (IC_{50}) of several standard-of-care chemotherapeutic agents used in TNBC along with Nutlin-3, an MDM2/p53 binding inhibitor across the panel of isogenic clones relative to parental controls. Once we have completed the analyses for a larger panel of drugs and the results are peer-reviewed, these data will be available to the scientific community.

5. CHANGES/PROBLEMS

Nothing to report at this time

6. PRODUCTS

Poster Presentations to Communities of Interest:

Shaver TM, Jin H, Tang L, and Pietenpol JA. "Targeting the p53 mutant-adpated state in triple-negative breast cancer." 8th International MDM2 Workshop, New Orleans, LA, November 2015.

Shaver TM, Jin H, Tang L, and Pietenpol JA. "Targeting the p53 mutant-adpated state in triple-negative breast cancer." 7th International p63/p73 Workshop, Boston, MA, April 2016.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

Name:	Jennifer Pietenpol, PhD
Project Role:	PI
Effort:	1.44 calendar months
Contribution to Project:	Oversight of all aspects of the project; design, interpretation and dissemination and reporting of results
Funding support:	DoD Award W81XWH-13-1-0287; NIH/NCI; Susan G. Komen Foundation; and Insight Pharmaceuticals

Name: Timothy Shaver
Project Role: Graduate Student
Effort: 12 calendar months
Contribution to Project: Involved in the design and conduct of all experiments performed and interpretation of data generated
Funding support: NIH/NCI F31 pre-doctoral NRSA Fellowship Grant

Name: Hailing Jin
Project Role: Senior Research Associate
Effort: 12 calendar months
Contribution to Project: Involved in the conduct of experiments performed
Funding support: DoD Award W81XWH-13-1-0287

8. SPECIAL REPORTING REQUIREMENTS

Nothing to report at this time.

9. APPENDICES

Not applicable

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